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—Original Article—

Effects of exposure to methylglyoxal on sperm motility and embryonic development after fertilization in mice

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Abstract. Methylglyoxal (MG) is a precursor for the generation of endogenous advanced glycation end-products involved in various diseases, including infertility. The present study evaluated the motility and developmental competence after *in vitro* fertilization of mouse sperm which were exposed to MG in the capacitation medium for 1.5 h. Sperm motility was analyzed using an SQA-V automated sperm quality analyzer. Intracellular reactive oxygen species (ROS), membrane integrity, mitochondrial membrane potential, and DNA damage were assessed using flow cytometry. The matured oocytes were inseminated with MG-exposed sperm, and subsequently, the fertilization and embryonic development *in vitro* were evaluated *in vitro*. The exposure of sperm to MG did not considerably affect the swim-up of sperm but resulted in a deteriorated sperm motility in a concentration-dependent manner, which was associated with a decreased mitochondrial activity. However, these effects was not accompanied by obvious ROS accumulation or DNA damage. Furthermore, MG diminished the fertilization rate and developmental competence, even after normal fertilization. Collectively, a short-term exposure to MG during sperm capacitation had a critical impact on sperm motility and subsequent embryonic development after fertilization. Considering that sperm would remain *in vivo* for up to 3 days until fertilization, our findings suggest that sperm can be affected by MG in the female reproductive organs, which may be associated with infertility.

Key words: Embryonic development, Methylglyoxal, Mitochondrial membrane potential, Reactive oxygen species, Sperm motility

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In Japan, approximately 15% of couples of reproductive age are infertile, with nearly half of them are associated with abnormalities in the male partner [1, 2]. Most of the cases of male infertility are caused by abnormal spermatogenesis and failure in sperm function. Decrease in male fertility has been linked to environmental factors, smoking, alcohol abuse, chronic stress, obesity and inflammation in the male reproductive system [3–6], which causes testicular dysfunction and poor sperm quality. Reproductive disorders are strongly associated with the overproduction of the reactive oxygen species (ROS) and oxidative stress. It has been reported that ROS generation occurs during the production of advanced glycation end-products (AGEs), and AGEs are largely accumulated in the reproductive tract and semen of patients with diabetes [7, 8]. Furthermore, in some male patients experiencing infertility, there is some possibility that sperm function would be affected in the female genital tracts after an ejaculation.

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AGEs are produced from carbonyl compounds such as glyoxal, methylglyoxal (MG), and 3-deoxyglucosone, as by-products during the glycation of proteins [9]. Carbonyl compounds are produced from the reducing sugars such as glucose as a by-product of the glycolytic pathway, and is strongly reactive with proteins [10, 11]. This glycation process generates ROS, which causes DNA damage and brings on the dysfunction of various proteins by cross-linking [12, 13]. In particular, MG reacts with a wide range of proteins and strongly promotes glycation when compared with glucose and other carbonyl compounds.

In patients with diabetes, it has been reported that the blood MG level is approximately 2 μM [7]. In an animal model, the blood MG level in hypertensive rats (SHR) was shown to increased with aging and was approximately 33.6 μM when compared with 14.2 μM in Wistar Kyoto rats (WKY) [14]. Regarding the effect of glycation on the reproductive systems, there have been several reports on studies using cells derived from the female reproductive organs and preimplantation embryos. Hajagos-Toth *et al*. [15] have evaluated the effect of antioxidants during the last trimester of pregnancy on the proliferation of rat myometrium cells and the ion channels, which were treated with 30 mM MG for 30 min. Furthermore, Hsuuw *et al*. [16] have investigated the apoptosis of mouse blastocysts following treatment with 200 μM MG for 3 h, considering increased MG levels

detected in patients with diabetes. On the other hand, the effect of glycation on testicular function has been investigated using murine Leydig cells and animal models of erectile dysfunction [17, 18]. In human, the ejaculated sperm from patients with diabetes demonstrate a decrease in motility and an increased AGE accumulation [19, 20]. Some reports indicate that these human sperm undergo increased DNA fragmentation, with deteriorated developmental potential to the blastocyst stage after *in vitro* fertilization [21, 22]. Nevin *et al.* have demonstrated that exposure of human sperm to 100 μM of MG induces cell membrane damage [23]. However, the influence of MG in sperm function has not been comprehensively investigated, and the fertilization and developmental potential of MG-exposed sperm remain unclear. In some male patients experiencing infertility, male germ cells including sperm are supposedly to be exposed to MG during various processes such as spermatogenesis in the testis, maturation and storage in the epididymis, and capacitation in the female genitalia. In the present study, the function of mouse sperm exposed to MG was investigated by evaluating several sperm parameters, oxidative stress, and DNA integrity. Furthermore, we examined the fertilization and embryonic development using MG-exposed sperm, which revealed that sperm with low MG-exposure levels could fertilize with oocytes normally but presented deteriorated developmental competence. Our findings suggest the hidden influence of the glycation-induced sperm damage on male infertility after fertilization.

Materials and Methods

Animals

All mice (ICR strain) were purchased from Kiwa Laboratory Animals (Wakayama, Japan) at 8 weeks of age and maintained in light-controlled and air-conditioned rooms. This research was performed in accordance with the recommendations in the Guidelines of Kindai University for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kindai University (Permit Number: KABT-31-024).

Sperm preparation and sperm motility analysis

Sperm collection procedures were performed as previously described [24]. In brief, spermatozoa were collected from the cauda epididymis of male mice. The sperm suspension was incubated in a modified human tubal fluid (mHTF) medium [25] with or without various concentrations of MG (Sigma-Aldrich, St. Louis, Missouri, USA) (300 μM, 500 μM, 1 mM, and 5 mM) for 1.5 h at 37ºC under 5% CO_2 in air. After incubation, the upper portion of the sperm suspension (the upper portion) was separated from the lower portion containing condensed sperm (the lower portion) after the incubation, and both portions of the sperm suspension after the incubation were manually evaluated for total sperm concentration and motile sperm rates using a Makler counting chamber (Seifi-Medical Instruments, Haifa, Israel) under a phase-contrast microscope (× 200). Furthermore, the upper portion and lower portion of the sperm suspension were analyzed using the SQA-V automated sperm quality analyzer (Medical Electronic Systems Ltd., Caesarea Industrial Park, Israel) [26]. The SQA-V simultaneously measured various parameters including motile sperm concentration (MSC), rapid progressive MSC (rapid PMSC), slow progressive MSC (slow PMSC), non-progressive MSC (non-PMSC), average velocity of motile sperm (velocity), and sperm motility index (SMI). SMI values are calculated based on the number of sperm and the type of motility.

Measurement of intracellular reactive oxygen species (ROS) and membrane integrity

ROS generation was assessed using CellROX® Green reagent (Molecular Probes, Eugene, OR, USA). It is a fluorescent probe that penetrates the cell and emits a more intense green fluorescence when oxidized by intracellular free radicals. To evaluate sperm membrane integrity, we measured the fluorescence of propidium iodide (PI), which is incorporated into the nuclei of dead cells. After the incubation for 1.5 h, the upper portion of the sperm suspension was incubated in mHTF containing 5 μM CellROX® Green and 1 μg/ml PI for 30 min at 37ºC, washed twice with cold phosphate-buffered saline (PBS), and analyzed using flow cytometry (FACS Calibur with CellQuest software, BD Biosciences, San Jose, CA, USA) [27] with fluorescence excited with 488 nm argon laser and detected with 530/30 nm BP (CellROX) and 670 nm LP (PI) emission filter, respectively. As a reference, sperm were incubated in mHTF containing 1 mM hydrogen peroxide. In total, 10,000 events were counted in each sample, and experiments were performed in triplicate.

Measurement of mitochondrial membrane potential (MMP)

MMP was measured using a JC-10 (Thermo Fisher Scientific, Waltham, MA, USA). This probe emits green or red-orange fluorescence for low or high mitochondrial potential, respectively. After the incubation for 1.5 h, the upper portion of the sperm suspension prepared as mentioned above, was incubated with mHTF containing 10 μg/ml JC-10 for 30 min at 37°C and analyzed by flow cytometry. MMP was represented as the ratio of red/green fluorescence excited with 488 nm argon laser and detected with 670 nm (red) and 530/30 nm (green) BP emission filters, respectively. As a reference, sperm were incubated in mHTF containing 1 mM hydrogen peroxide. In total, 10,000 events were counted in each sample, and experiments were were performed in triplicate.

Sperm chromatin structure assay (SCSA)

Sperm DNA damage was evaluated by SCSA using a metachromatic fluorescence probe, acridine orange (AO) [28]. The upper portion of the sperm suspension prepared as previously described, was incubated with TNE buffer (Tris-HCl 0.01 M, NaCl 0.15 M, EDTA 1 mM, and distilled water, pH 7.4), followed by the addition of an acid detergent solution (HCl 0.08 M, NaCl 0.15 M, and Triton X-100 0.1% in distilled water, pH 1.2). After 30 sec, the samples were stained with AO solution (citric acid 0.1 M, Na_2HPO_4 0.2 M, EDTA 0.001 M, NaCl 0.15 M, and AO 6 μg/ml in distilled water, pH 6.0) for 5 min at 37°C. Finally, samples were analyzed by flow cytometry with fluorescence excited with 488 nm argon laser and detected with 630–650 nm (red) emission filter, respectively. As a reference, sperm were incubated in mHTF containing 1 mM hydrogen peroxide. In total, 10,000 events were counted in each sample, and experiments were repeated at six times.

In vitro fertilization and culture

In vitro fertilization was performed according to a previously

described method [24]. In brief, sperm were collected as previously described. Oocytes surrounded by cumulus cells (COCs) were collected from the excised oviducts of superovulated female mice. After 1.5 h of incubation, the upper portion of the sperm suspension was added to the mHTF-containing COCs. Six hours after insemination, morphologically normal fertilized oocytes were recovered and cultured in modified potassium simplex optimized medium (mKSOM) at 37°C under 5% CO_2 in air. Embryos at the 2- cell, 4- cell, 8-cell, morula and blastocyst stages were observed at 24, 48, 72, 84, and 96 h post-insemination (hpi), respectively. The blastocysts were treated with acidified Tyrode's solution (FUJIFILM Irvine Scientific, Saitama, Japan) to remove the zona pellucida and were rinsed in PBS containing 0.3% polyvinyl pyrrolidone (0.3% PVP/PBS). Thereafter, they were fixed in 4% paraformaldehyde (PFA; FUJIFILM Wako Chemicals, Osaka, Japan) for 15 min, rinsed in 0.3% PVP/PBS, and permeabilized using PBS containing 0.2% TritonX-100 (Sigma-Aldrich, St. Louis, Missouri, USA) for 15 min. Finally, they were stained with 1.5 µg/ml 4',6-diamidino-2-phenylindole (VECTASHIELD Mounting Medium with DAPI; Vector Laboratories, Burlingame, California, USA). The total cell number of blastocysts was counted using a fluorescence microscope (AF6500; Leica Microsystems, Wetzlar, Germany) equipped with a UV filter (Ex 360 nm).

Statistics

Data are expressed as the mean ± standard deviation (SD). Sperm motility was assessed by one-way analysis of variance (ANOVA) using Tukey's multiple range test. Intracellular ROS and sperm membrane integrity, MMP and SCSA were compared using the Student's *t*-test. *In vitro* fertilization and embryonic development were evaluated using the Chi-square test. Statistical analyses were performed using StatView version 5.0 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Sperm motility

Table 1 summarizes the results of sperm motility assessments. After incubation for 1.5 h with or without MG, the sperm motility was compared between the upper portion and lower portion. In the upper portion, the total sperm concentration of non-treated sperm was 80.0 million/ml, and that of sperm treated with MG did not significantly differ at 300 µM, 500 µM, and 1 mM (86.0, 75.0, and 69.0 million/ml, respectively) and exhibiting a significant difference at 5 mM (57.0 million/ml). In contrast, the total sperm concentration of the lower portion was ranged between 228.0 and 268.0 million/ml, demonstrating no significant difference. In the sperm suspension, the motile sperm rate of the non-treated upper portion of sperm suspension was 67.9%, and that of the upper portion treated with MG at 300 µM MG did not differ significantly (61.6%). On the other hand, the motile sperm rate of the upper portion treated with MG at 500 µM, 1 mM and 5 mM significantly declined (49.3%, 35.9%, and 2.7%, respectively). In particular, most of the sperm lost its motility after treatment with 5 mM MG. For the lower portion of the sperm suspension, the motile sperm rate did not differ between non-treated and MG-treated sperm at 300 µM and 500 µM (37.2%, 39.4%, and 33.6%, respectively), but decreased significantly after treatment with MG at 1 mM and 5 mM MG in a concentration-dependent manner (19.8% and 1.0%, respectively). The more detailed parameters such as progressive motility and velocity of sperm were measured using SQA-V. In the upper portion, the gross MSC was remarkably reduced following MG treatment when compared with the lower portion of the sperm suspension. MSCs of MG-treated sperm at 300 µM to 1 mM significantly decreased less than half (18.7, 18.7, and 10.8 million/ml, respectively) when compared with the non-treated sperm (42.5 million/ml). Based on parameters of MSC, it was observed that rapid PMSC of MG-treated sperm at 300 µM and 500 µM did

Table 1. Motility parameters of the sperm exposed to methylglyoxal

	Total sperm concentration (10^6/ml)*	Motile sperm rate (%) *	Motile sperm concentration (10^6/ml)	Rapid progressive motile sperm conc (10^6/ml)	Slow progressive motile sperm conc (10^6/ml)	Non progressive motile sperm conc (10^6/ml)	Velocity (µm/sec)	Sperm motility index (SMI)
Upper portion of the sperm suspension								
0 µM	80.0 ± 11.2 [a]	67.9 ± 2.4 [a]	42.5 ± 7.1 [a]	4.7 ± 0.5 [a]	21.7 ± 6.5 [a]	16.1 ± 0.3 [a]	7.3 ± 1.2 [a]	140.0 ± 32.9 [a]
300 µM	86.0 ± 2.6 [a]	61.6 ± 1.3 [a]	18.7 ± 8.0 [b]	4.6 ± 0.3 [a]	5.7 ± 4.8 [b]	8.1 ± 3.1 [b]	6.0 ± 0.1 [a]	94.0 ± 8.6 [b]
500 µM	75.0 ± 4.1 [a]	49.3 ± 1.3 [b]	18.7 ± 6.0 [b]	4.7 ± 0.4 [a]	6.0 ± 4.2 [b]	8.0 ± 1.5 [b]	6.7 ± 0.6 [a]	93.3 ± 10.1 [b]
1 mM	69.0 ± 5.7 [a]	35.9 ± 1.5 [b]	10.8 ± 0.4 [c]	2.8 ± 0.3 [b]	0.4 ± 0.1 [b]	7.5 ± 0.3 [b]	2.7 ± 0.6 [b]	52.7 ± 3.1 [c]
5 mM	57.0 ± 2.5 [b]	2.7 ± 1.6 [c]	3.8 ± 3.8 [c]	0.2 ± 0.2 [c]	0.0 ± 0.0 [b]	3.6 ± 3.1 [c]	0.7 ± 0.6 [c]	2.3 ± 2.0 [d]
H_2O_2	64.0 ± 4.3	3.6 ± 1.8	0.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Lower portion containing condensed sperm								
0 µM	253.0 ± 11.4 [e]	37.2 ± 0.7 [e]	67.3 ± 2.4 [e]	12.9 ± 1.6 [e]	35.1 ± 1.0 [e]	19.3 ± 0.3 [e]	9.0 ± 0.0 [e]	252.7 ± 12.1 [e]
300 µM	248.0 ± 7.3 [e]	39.4 ± 1.9 [e]	47.2 ± 17.4 [e]	5.4 ± 0.7 [f]	25.2 ± 13.4 [e]	16.6 ± 3.9 [e]	7.0 ± 1.0 [e]	157.0 ± 52.0 [f]
500 µM	268.0 ± 9.9 [e]	33.6 ± 0.6 [e]	54.4 ± 19.8 [e]	6.1 ± 1.0 [f]	30.6 ± 13.9 [e]	17.8 ± 4.9 [e]	7.7 ± 1.2 [e]	164.7 ± 66.6 [f]
1 mM	228.0 ± 7.8 [e]	19.8 ± 1.3 [f]	61.3 ± 30.1 [e]	4.6 ± 0.7 [f]	32.5 ± 25.2 [e]	24.2 ± 11.0 [e]	6.3 ± 1.5 [f]	183.0 ± 76.2 [f]
5 mM	228.0 ± 5.9 [e]	1.0 ± 0.5 [g]	9.1 ± 8.2 [f]	0.3 ± 0.4 [g]	0.2 ± 0.3 [f]	8.5 ± 7.6 [f]	0.7 ± 0.6 [g]	12.7 ± 11.0 [g]
H_2O_2	240.0 ± 6.7	0.1 ± 0.0	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0

* Sperm were manually evaluated for total sperm concentration and motile sperm rates using a Makler counting chamber. Values for each parameter are presented as the mean ± standard deviation (SD). Different letters indicate significant differences (a–d, e–g) ($P < 0.05$).

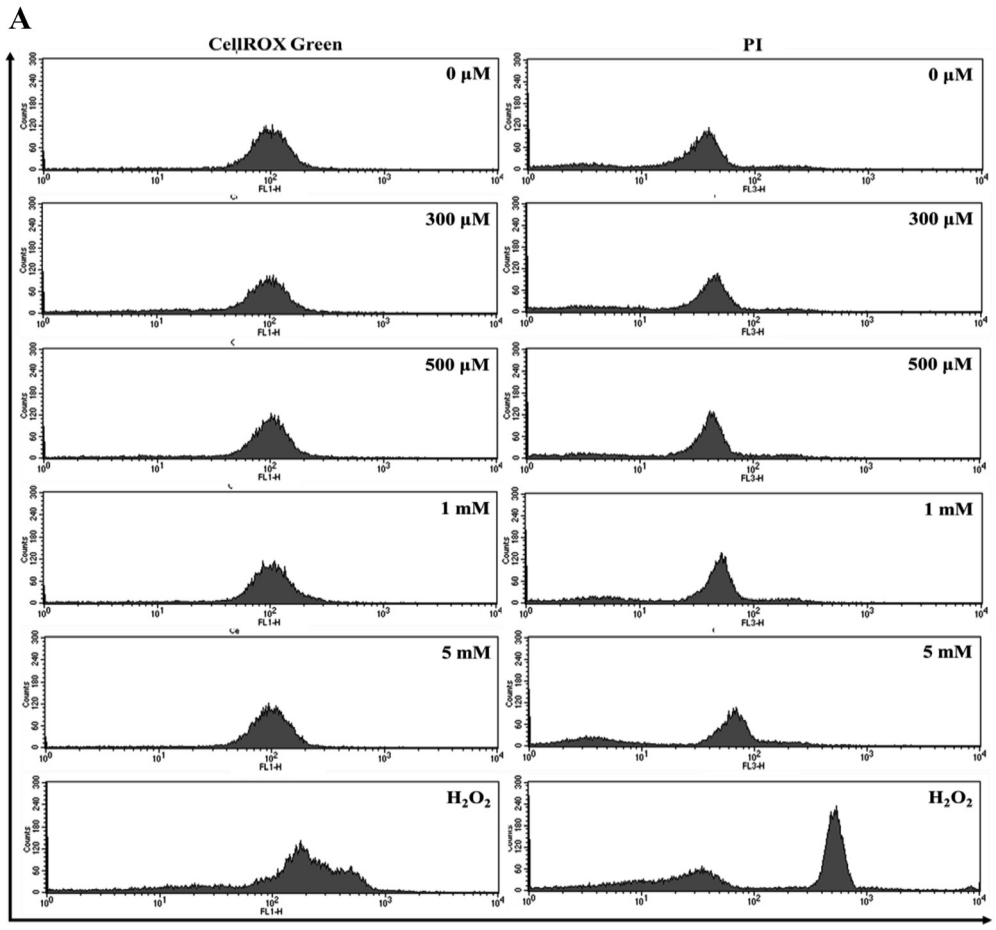

Fig. 1. Reactive oxygen species (ROS) accumulation and membrane integrity of methylglyoxal (MG) -exposed sperm analyzed by flow cytometry. Sperm were treated with MG at various concentrations for 1.5 h. Intracellular ROS levels were detected using CellROX Green reagent, and the membrane integrity was analyzed by propidium iodide (PI) staining. (A) Exposure of sperm to MG dose not induce intracellular ROS accumulation regardless of its concentrations, whereas 1 mM hydrogen peroxide treatment obviously induces intracellular ROS accumulation. On the contrary, the PI intensity increase moderately in a concentration-dependent manner. The PI intensity in 1 mM hydrogen peroxide treated sperm is higher than that observed in any MG-treated groups. (B, C) Flow cytometry plots using CellROX Green/PI staining (B) and the percentage of PI (–)/CellROX (+) fraction of MG-treated sperm to that of non-treated sperm (control) (C). Intracellular ROS accumulation in sperm treated with lower MG concentrations (300 µM, 500 µM) is equivalent to the control, but it gradually increases when exposed to higher concentrations (1 mM, 5 mM). Sperm exposed to 1 mM hydrogen peroxide could not be measured owing to the absent of PI (–) fraction. * Asterisks indicate significant differences from the control ($P < 0.05$).

not differ, decreasing significantly in MG-treated sperm at 1 mM and 5 mM. In contrast, slow PMSC and non-PMSC were more strongly affected by the MG treatment. Regarding PMSC, 5 mM of MG demonstrated a harmful effect on sperm motility. The velocity was 7.3 µm/sec in non-treated sperm and remained unaffected in MG-treated sperm at 300 µM and 500 µM (6.0 and 6.7 µm/sec, respectively). However, the velocity was significantly decreased at 1 mM and 5 mM (2.7 and 0.7 µm/sec, respectively). The SMI of MG-treated sperm, calculated using these parameters of SQA-V, significantly decreased in a concentration-dependent manner. For sperm treated with 300 µM and 500 µM MG, the SMI was 70% that of non-treated sperm; treatment with 1 mM MG drastically decreased SMI to one-third. For 5 mM MG-treated sperm, the SMI revealed an extremely low value. In the lower portion, the effect of MG on sperm motility was similar but tended to be smaller than that of sperm in the upper portion.

Intracellular ROS and membrane integrity

To investigate the membrane damage of the upper and lower portions of sperm, PI staining was performed. In the sperm suspension, the PI intensity of the lower portion was stronger than that of the upper portion of sperm in an MG concentration-dependent manner (Supplementary Fig. 1: online only), indicating that the lower portion of sperm were more considerably damaged. The following experiments were performed using only the upper portion of sperm suspension. The accumulation of intracellular ROS and membrane damage in sperm were assessed using CellROX® Green and PI, respectively. As a result, MG exposure to sperm did not affect intracellular ROS accumulation regardless of its concentration, whereas exposure to 1 mM hydrogen peroxide to sperm induced intracellular ROS

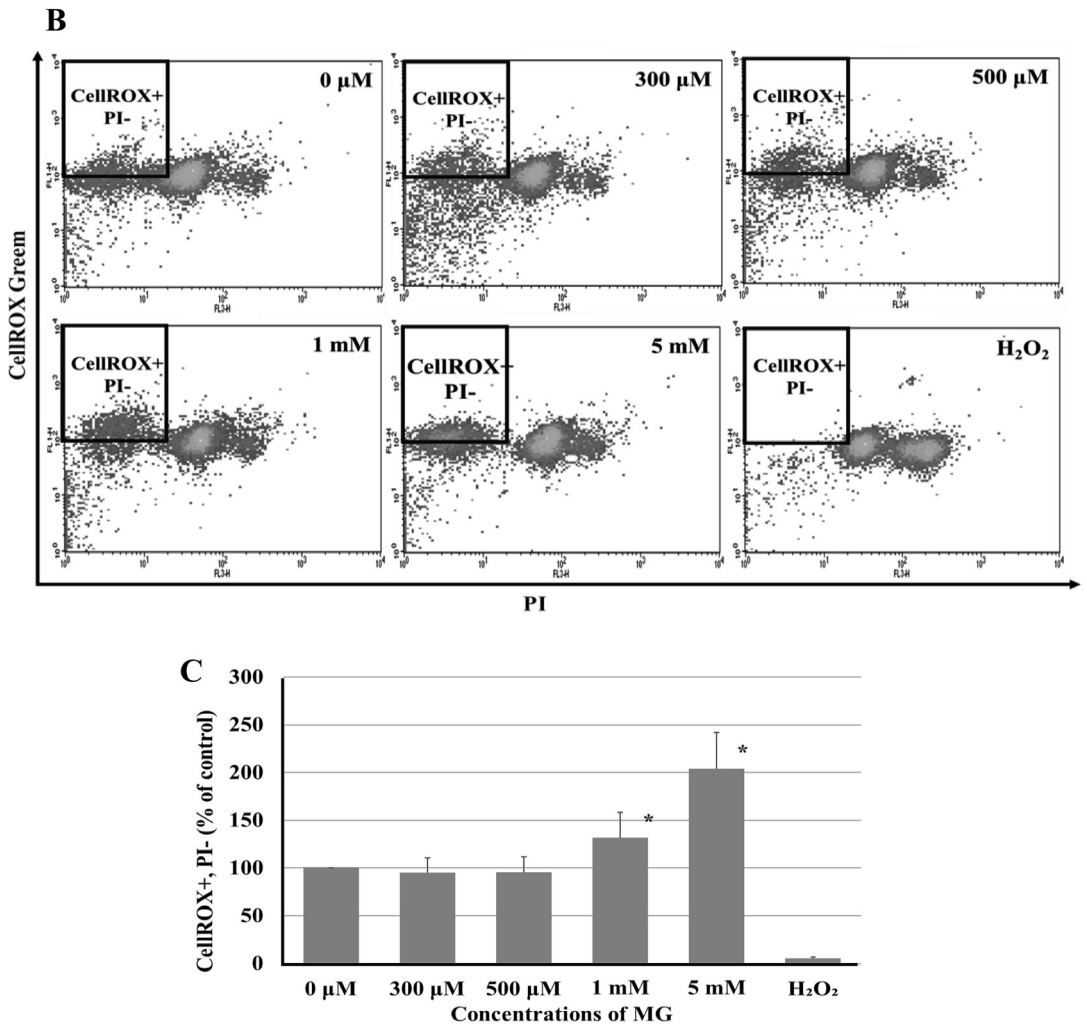

Fig. 1.

accumulation (Fig. 1A). In contrast, the fluorescence intensity of PI nevertheless increased moderately in 300 μM to 1 mM MG-treated sperm and more strongly in 5 mM MG-treated sperm when compared with non-treated one. In the sperm treated with 1 mM hydrogen peroxide, the PI intensity was higher than that observed in any group treated with MG. Although a moderate increase of PI intensity was observed in 300 μM to 1 mM MG-treated sperm, the MG treatment at those conditions did not affect the sperm motility, as shown in Table 1; hence, the moderate PI-positive sperm were not dead. However, the sperm treated with 5 mM MG showed a further increase in PI intensity, with loss in motility. The population of PI-negative/CellROX-positive sperm was increased in the higher MG concentration (1 mM, 5 mM) groups when compared with the lower MG concentration groups (300 μM, 500 μM), indicating that high MG concentration induce intracellular ROS accumulation in live sperm (Figs. 1B and C).

Mitochondrial membrane potential (MMP)

As mitochondrial integrity is highly related to sperm viability, we examined whether MG induces mitochondrial dysfunction by evaluating MMP using JC-10 (Figs. 2A and B). Flow cytometric analysis revealed that the ratio of sperm with high MMP (red/green) declined considerably in MG-treated sperm in a concentration-dependent manner. However, sperm treated with 5 mM MG demonstrated a moderate decline in MMP when compared with sperm treated with lower MG owing to the bimodal fluorescence distribution in the right half region.

Sperm chromatin structure assay (SCSA)

Sperm DNA damage following MG exposure was evaluated using SCSA (Fig. 3A). The DNA fragmentation index (DFI) was lower in the 300 μM to 1 mM MG-treated and 1 mM hydrogen peroxide-treated sperm than in non-treated sperm; however, no significant difference was observed. In the case of sperm treated with 5 mM MG, the DFI was the same as that in non-treated sperm (Fig. 3B).

In vitro fertilization and embryonic development

To examine the effect of MG-exposed sperm on fertilization and

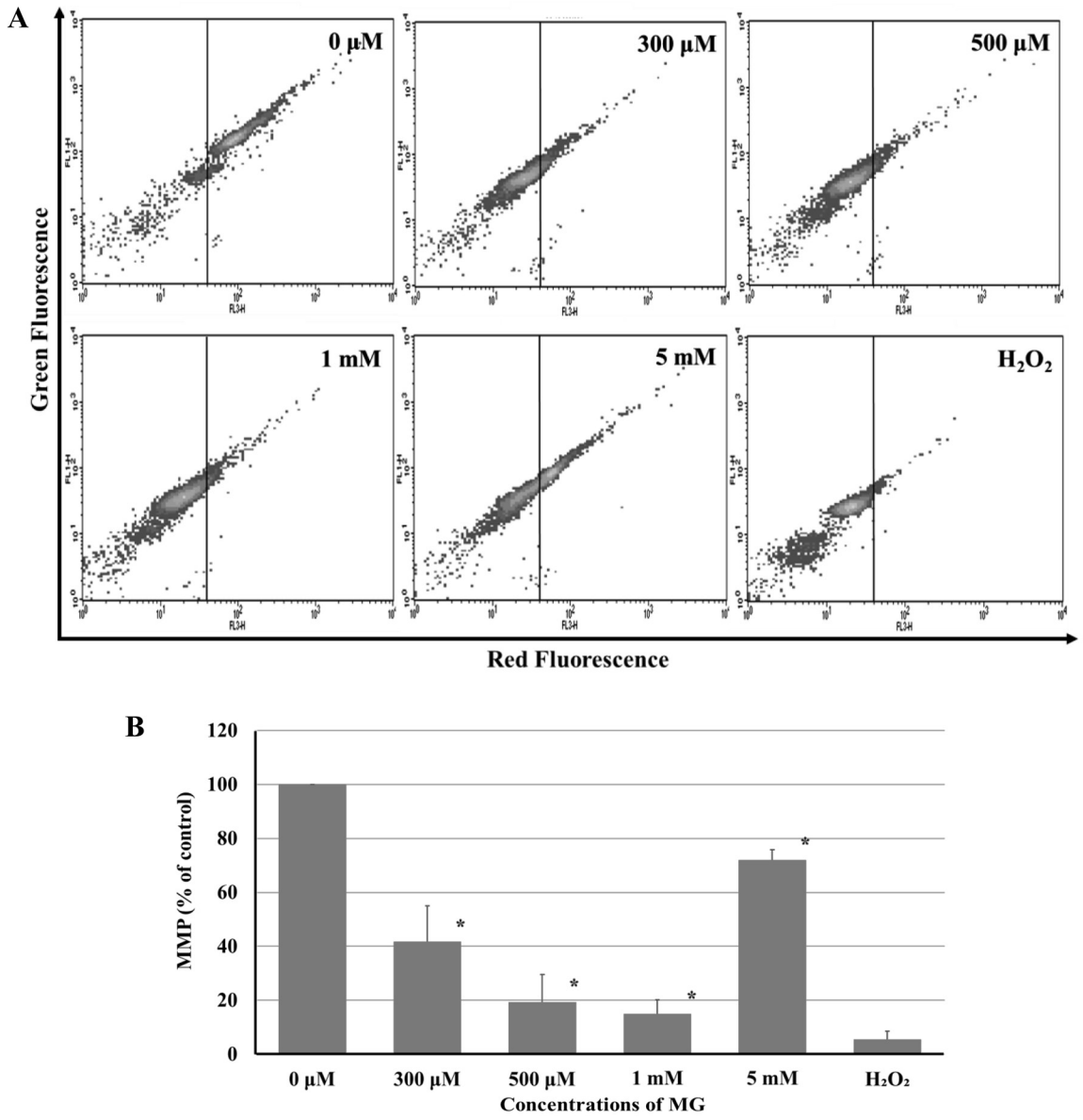

Fig. 2. Mitochondrial membrane potential (MMP) assay of the methylglyoxal (MG) -exposed sperm using JC-10 and flow cytometry. (A) The right half region in each dot plot diagram includes sperm with high MMP (HMMP), and the left half region indicates sperm with low MMP (LMMP). The population of sperm with LMMP increases following treatment with MG- or hydrogen peroxide-treatment. (B) The MMP (HMMP/LMMP) percentage of MG-treated sperm to that of non-treated sperm (control). Values are presented as the mean ± standard deviation (SD). * Asterisks indicate significant differences from the control ($P < 0.05$).

embryonic development, we performed *in vitro* fertilization. Table 2 summarizes the results of *in vitro* fertilization experiments. Six hours after insemination, the majority of the oocytes inseminated with non-treated sperm contained 2 pronuclei (2PN; 91.9%). However, the exposure to sperm at 300 µM, 500 µM, and 1 mM MG during incubation significantly decreased normal fertilization (2PN) in a concentration-dependent manner (78.3%, 64.3%, and 44.9%, respectively), owing to the prevention of sperm penetration or unidentifiable pronuclei (0PN). Table 3 summarizes the results of the embryonic development of fertilized oocytes. Overall, more than 80% of oocytes fertilized with non-treated and 300 µM MG-treated sperm developed into blastocysts (86.5% and 80.9%, respectively). On the other hand, the developmental ability of oocytes fertilized with sperm treated at 500 µM and 1 mM MG, to the blastocyst stage, significantly decreased in a concentration-dependent manner (51.4% and 35.0%, respectively). There was no morphological difference between the blastocysts using MG-treated and the non-treated sperm (Fig. 4A). The total number of cells was 46.2 ± 11.3 (n = 19), 44.6

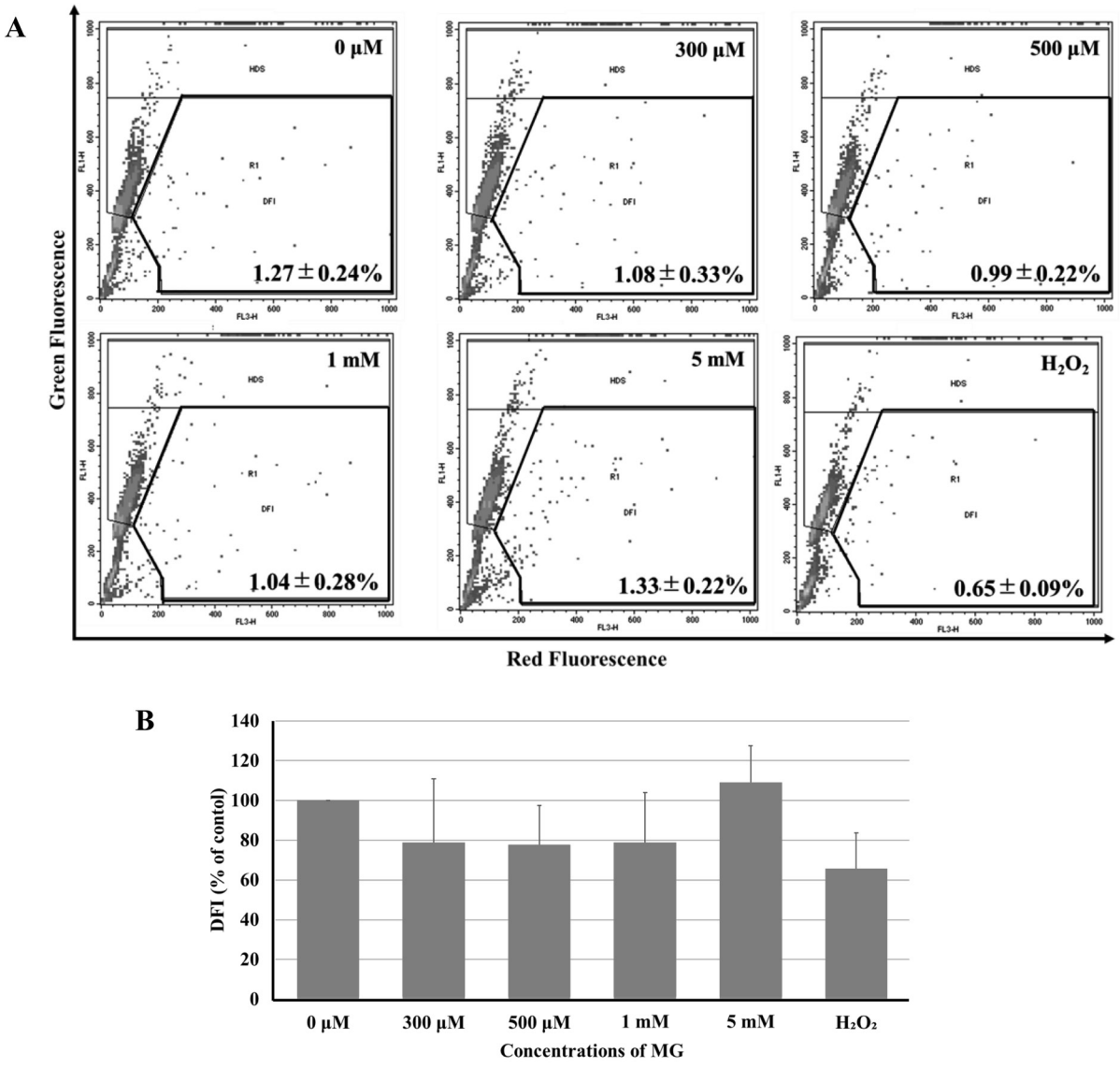

Fig. 3. DNA fragmentation analysis of the methylglyoxal (MG)-exposed sperm by the sperm chromatin structure assay (SCSA). (A) Dot plot cytograms using green fluorescence (intact DNA) and red fluorescence (fragmented single-stranded DNA) channels indicate sperm DNA fragmentation. The sperm with fragmented DNA are depicted in the thick-frame presented as DNA fragmentation index (DFI). The DFI are indicated as the mean ± percentage standard deviation (SD%). (B) The percentage of the DFI of MG-treated sperm to that of non-treated sperm (control). There is no difference between MG-treated and non-treated sperm.

± 13.9 (n = 12), 42.6 ± 7.2 (n = 10) and 42.1 ± 13.9 (n = 8) for the blastocysts generated using sperm exposed to 0 µM, 300 µM, 500 µM, and 1 mM MG, respectively (Fig. 4B), with there was no significant difference observed among them.

Discussion

In *in vitro* studies, cytotoxicity of MG has been investigated using cultured cells by exposure to MG concentrations ranging between 300 µM and 1 mM, for 1–3 h [29, 30]. For example, in a study investigating smooth muscle contraction in hypertensive model rats, mesenteric artery cells were treated with 420 µM MG for 30 min [29]; and to assess long-term exposure, was performed for 3 days with 42 µM, which corresponds to the blood MG level in hypertensive rats [30]. Human sperm can survive for longer than 2 days in the female genital tracts to wait for fertilization to occur [31]; however, it is difficult to retain mouse sperm for 3 days in an *in vitro* environment, and hence, it is crucial to estimate the effects of MG under the examined conditions. Our results revealed that MG affected sperm function in a concentration-dependent manner, which resulted in a decrease in sperm motility (Table 1). Sperm motility was associated with the decreased mitochondrial activity (Fig. 1);

Table 2. *In vitro* fertilization of oocytes inseminated with sperm exposed to methylglyoxal

MG	Number of oocytes used	Number (%) of oocytes with			
		2PN *	1PN	3PN	0PN
0 μM	124	114 (91.9) [a]	2 (1.6)	1 (0.8)	7 (5.6)
300 μM	60	47 (78.3) [b]	5 (8.3)	0 (0.0)	8 (13.3)
500 μM	112	72 (64.3) [c]	0 (0.0)	1 (0.9)	39 (34.8)
1 mM	89	40 (44.9) [d]	3 (3.4)	0 (0.0)	46 (51.7)

* Only oocytes with two pronuclei were used for subsequent *in vitro* culture experiments. Different letters indicate significant differences ($P < 0.05$). MG, methylglyoxal; 2PN, 2 pronuclei; 1PN, one pronucleus; 3PN, three pronuclei; 0PN, absence of pronuclei.

Table 3. Embryonic development of oocytes fertilized with sperm exposed to methylglyoxal

MG	Number of fertilized oocytes used *	Number (%) of fertilized oocytes developed to				
		2-cell	4-cell	8-cell	Morula	Blastocyst
0 μM	111	111 (100.0)	106 (95.5)	104 (93.7)	102 (91.9)	96 (86.5) [a]
300 μM	47	46 (97.9)	46 (97.9)	46 (97.9)	45 (95.7)	38 (80.9) [a]
500 μM	72	65 (90.3)	61 (84.7)	53 (73.6)	46 (63.9)	37 (51.4) [b]
1 mM	40	36 (90.0)	30 (75.0)	25 (62.5)	23 (57.5)	14 (35.0) [c]

* Only oocytes with two pronuclei were used for *in vitro* culture. Different letters indicate significant differences ($P < 0.05$).

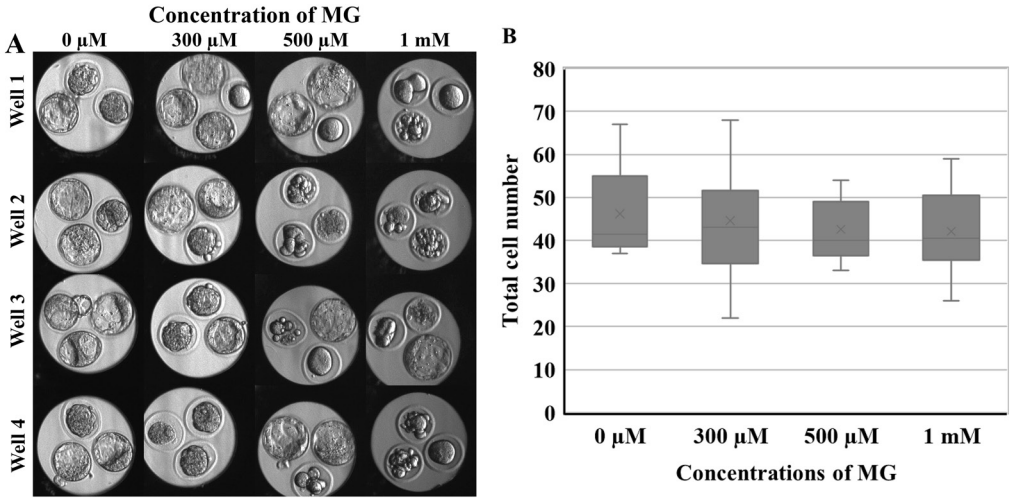

Fig. 4. The morphology and total cell number of blastocysts using sperm exposed to methylglyoxal (MG). (A) *In vitro* development of the fertilized eggs using sperm exposed to MG. In MG-treated groups, the degenerated eggs increased in a concentration-dependent manner. However, no morphological difference can be observed among the blastocysts using MG-treated groups and control. Magnification: 150 ×. (B) The total cell number of blastocysts using MG-treated and non-treated sperm (control). The x signs near the median lines of each graph represent the mean value. There is no difference between MG-treated groups and control.

however, it was not accompanied by obvious ROS accumulation or DNA damage (Figs. 2 and 3). In addition, MG deteriorated not only the fertilization rate but also the developmental competence of oocytes that were normally fertilized (with 2PN) (Tables 2 and 3).

Regarding the sperm preparation, the sperm concentration in the upper portion tended to gradually decrease in a concentration-dependent manner, and a significant difference was observed in the 5 mM MG-treated sperm; this indicated that MG did not considerably affect the swim-up of sperm itself. On the contrary, in the lower portion, it was presumed that the sperm concentration did not increase over a certain level, probably owing to spontaneous sedimentation. In contrast, in mouse sperm, MG exposure significantly deteriorated the motile sperm rate and parameters of MSC. Particularly, the major subpopulation of slow PMSC was remarkably reduced, resulting in decreased sperm motility index (SMI). Previous reports have shown that MG impairs cell viability in human retinal pigment epithelial

cells and bovine retinal pericytes [32, 33] and reduces the viability of human sperm [23]. Therefore, to investigate the cause of decreased sperm motility following MG exposure, we examined the integrity of the sperm membrane. Following PI staining, we observed that the PI intensity of sperm in the lower portion was stronger than that of the upper portion of sperm suspension in an MG concentration-dependent manner (Supplementary Fig. 1), suggesting that the lower portion of sperm suspension was damaged more severely. In the upper portion of the sperm suspension exposed to 1 mM hydrogen peroxide, the PI intensity was remarkably increased, and CellROX assay showed an accumulation of ROS in those sperm. On the other hand, MG treatment resulted in only a marginal increase in PI intensity, implying that the cell membrane was moderately damaged (Fig. 1A). According to the motile sperm rate in Table 1, sperm was mostly unaffected by such MG concentrations, except for 5 mM MG. The PI intensity in 5 mM MG-exposed sperm was only marginally stronger than that in the lower MG-exposed groups; however, it remains unclear whether such a slight change of the PI intensity could be a direct cause of decreased motility. In contrast, CellROX assay showed no ROS accumulation even in 5 mM MG-exposed sperm, suggesting that ROS was not a direct factor contributing to decreased sperm motility. Interestingly, in a subpopulation with the PI-negative sperm, no ROS accumulation was observed in 300 μM and 500 μM MG-exposed groups, whereas ROS was significantly accumulated in 1 mM and 5 mM MG-exposed groups (Figs. 1B and C). Sperm exposed to 1 mM hydrogen peroxide could not be measured because of the absent of the PI (–) fraction. These results indicate that even in the motile sperm, the accumulation of ROS increased in a concentration-dependent manner with concentrations exceeding 1 mM, which may affect the fertility and developmental ability of the embryos using these sperm.

Flagellar movement of sperm is one of the major determinants of male fertility and is regulated by the ATP supply generated by glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). These metabolic pathways are adjusted depending on the maternal reproductive environment, and the oxidative phosphorylation in mitochondria is essential for sperm linear motility [34] and is governed by the MMP [35]. Our results demonstrated that MG did not significantly affect the sperm cell membrane damage and sperm viability but decreased MMP in a concentration-dependent manner, resulting in the deterioration of mitochondrial activity (Fig. 2B). Therefore, it was considered that reduced MMP affected the motile sperm rate and rapid PMSC. Furthermore, the ATP production and mitochondrial DNA transcription in sperm are impaired by an increased ROS [36], but decreased MMP without ROS accumulation in sperm treated with lower MG concentrations may indicate that certain factors other than oxidative stress affect MMP. It remains unclear why most sperm exposed to 5 mM MG are suddenly immobilized despite the marginal decrease in MMP.

MG elicits various harmful effects such as ROS production, DNA damage, and protein dysfunction owing to cross-linking [10, 11] during glycation reactions. For example, in bovine aortic endothelial cells, certain millimolar concentrations of MG can induce oxidative stress, causing apoptosis [37, 38]. However, neither accumulation of ROS (Fig. 1A) nor DNA fragmentation (Figs. 3A and B) in sperm was observed despite treatment with 5 mM MG. As mouse sperm is rather homogenous, robust, and stable when compared with human [39], the MG conditions applied here did not possibly induce DNA damage.

To the best of our knowledge, for the first time, we observed that MG-treated sperm affected embryonic development after fertilization. The fertilization rate gradually declined depending on the MG concentration (Table 2), probably owing to the diminished sperm motility (Table 1). Interestingly, even in the normally fertilized oocytes with 2PN, their developmental potential was impaired in a concentration-dependent manner (Table 3). On employing 300 μM MG-exposed sperm used, despite the normal fertilization rate of the oocytes being significantly reduced, the fertilized oocytes retained their developmental potential when compared with the control one. On the other hand, when oocytes were fertilized using sperm exposed to 500 μM or higher MG concentrations, their embryonic development was considerably reduced. These results suggest that sperm exposed to 300 μM of MG presented decreased its motility owing to a mitochondrial hypofunction, but still retained the post-fertilization developmental potential. Notably, although sperm exposed to a higher concentration of MG could produce seemingly normal eggs, they might have resulted in the impairment of the subsequent embryonic development, probably due to a certain level of ROS accumulation or other protein dysfunction in respective sperm. To date, it has been reported that oxidative stress in sperm affects epigenetic reprogramming and may affect embryogenesis [40, 41]. Alternatively, MG is highly reactive with lysine and arginine residues and is involved in epigenetic regulation as a histone mark [42]. Sperm chromatin is consists of arginine-rich protamine [43] and a small number of histones [44], and hence, MG could affect the post-fertilization epigenomic function. Herein, we compared the developmental potential of fertilized eggs produced by *in vitro* fertilization, but from a clinical standpoint; the developmental ability of the eggs fertilized with MG-exposed immotile sperm by intracytoplasmic sperm injection (ICSI) will provide useful information. Furthermore, as no difference was observed in the morphology and the total cell number of the blastocysts regardless of the MG treatment, it is necessary to evaluate detailed characteristics of the developed embryos and clarify the post-implantation development by transferring blastocysts into the uterus.

In this study, we hypothesized that short-term exposure of mature sperm to MG mimics the environmental effects of sperm in the maternal uterus and oviducts on sperm. In bovine female reproductive tissues, the MG concentration in the follicular fluid is 30 μM, correlating with the serum content [45]; in hypertensive rats (SHR), the blood MG level is approximately 33.6 μM [14]. In terms of clinical characteristics, patients with diabetic and/or polycystic ovarian syndrome show higher blood MG levels (approximately 2 μM) than healthy individuals and, are therefore considered more susceptible to glycation reactions *in vivo* [7, 46]. Consequently, in patients with diabetes, high AGE levels in the uterine cavity impair the endometrial function and result in embryonic implantation incompetence [47]. Therefore, when the MG concentration is high in the female genital tracts, sperm can be affected either its motility or fertility while sperm are retained until fertilization. Our study demonstrated that sperm motility and their physiological phenomena were affected following exposure to MG in a concentration-dependent manner in

the range of 300 μM-5 mM. Accordingly, the effects of chronic MG exposure on spermatogenesis need to be investigated using an *in vitro* induction system of spermatogenesis by employing spermatogonial stem cells [48, 49], elucidating the pathological influence of MG on male infertility.

In summary, we demonstrated that short-term exposure of sperm to MG affected sperm motility and subsequent embryonic development. Our findings suggest that the motility, fertilization ability and embryonic developmental competence of sperm can be affected by MG in the female reproductive organs even for short periods of up to 3 days. Therefore, MG and AGEs, as biomarkers of the maternal environment, may be potential therapeutic targets for improving fertility in sterile couples experiencing infertility.

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